

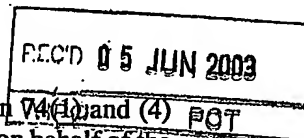


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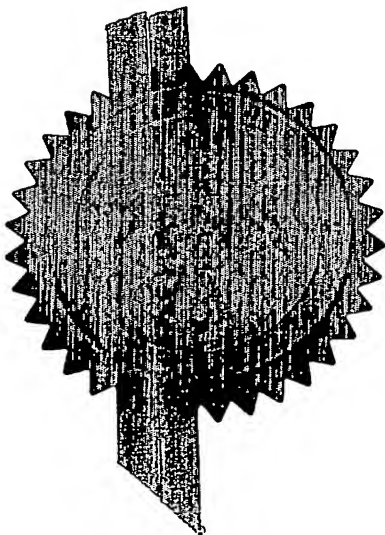


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19 APR 2002

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1. Your reference	444.77693/000		
2. Patent application number (The Patent Office will fill in this part)	0209007.4		
3. Full name, address and postcode of the or of each applicant (underline all surnames)	Norferm DA Vasabomen PO Box 8005 N-4003 Stavanger Norway Patents ADP number (if you know it) 7836265002 If the applicant is a corporate body, give country/state of incorporation Norway		
4. Title of the invention	Product		
5. Name of your agent (if you have one)	Frank B. Dehn & Co.		
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	179 Queen Victoria Street London EC4V 4EL		
Patents ADP number (if you know it)	166001 ✓		
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Description	9
Claim(s)	1
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Statement of inventorship and right to grant of a patent (Patents Form 7/77)	-
Request for preliminary examination and search (Patents Form 9/77)	-
Request for substantive examination (Patents Form 10/77)	-
Any other documents (please specify)	

Copies of GB0203306.6 and 0203307.4 ✓

11.

I/We request the grant of a patent on the basis of this application.

Signature  Date 19th April 2002
Frank B Dehn & Co

12. Name and daytime telephone number of person to contact in the United Kingdom

Julian Cockbain
020 7206 0600

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Product

5 The present invention relates to the use as a
microorganism growth substrate of a bacterial biomass,
in particular a biomass, herein termed a "bacterial
extract", deriving at least in part from a bacterial
culture comprising a methanotrophic bacterium.

10 Microorganisms are frequently grown on commercial
and laboratory scales, for example to produce desired
substances from bacterial strains which either naturally
produce such substances or have been genetically
modified so as to produce such substances, or so as to
determine the nature of a bacterial contamination, etc.
15 For these purposes the microorganisms require nutrients
and in this regard it is conventional to use yeast, meat
or plant extracts which are widely available
commercially, e.g. MRS (De Mann, Rogosa and Sharpe), PCA
(Plate Count Agar), VRBD (Violet Red Bile Dextrose
20 Agar), YM Agar (Yeast Mould Agar), Baird-Parker Agar
Base, VRB Agar (Violet Red Bile Agar), XLD Agar (Xylose
Lysine Deoxycholate Agar), CASO (Casein-peptone Soybean-
peptone), TSB (Tryptic Soy Broth) and NB (Nutrient
Broth). Yeast extract growth substrates (contained for
25 example in MRS, VRBD, VRB Agar, PCA, Baird-Parker Agar
Base, and XLD Agar) are available commercially from
Merck and Difco among others. Such yeast extracts are
commonly produced using biomass from yeast cultures
which has been allowed to autolyse, i.e. enzymes
30 naturally occurring within the yeast cells act to break
down the cells after cell death. Autolysis of yeast is
generally slow and several days may be needed before a
suitable degree of digestion is achieved. Accordingly,
additives which act as autolysis initiators or
35 stimulators, e.g. thiol agents, are generally used to
accelerate the autolysis process. The use of such
additives of course adds to the costs of commercial

production of yeast autolysates. To the resulting autolysates, extra nutrients may be added to optimize cell growth for particular microorganisms and indeed library deposits of microorganisms will generally specify which growth medium is most suitable for the deposited organism.

Since different microorganisms have different nutritional needs there is of course an ongoing need for alternative and improved microorganism growth media, particularly for growth media effective for growing those microorganisms which are challenging to grow in vitro (e.g. lactobacilli) and for "broad spectrum" growth media which may be suitable for use with unknown microorganisms.

We have now surprisingly found that particularly effective microorganism growth media may be produced using the biomass harvested from a culture medium comprising methanotrophic bacteria, e.g. biomass produced as described in WO 01/60974.

Viewed from one aspect therefore the present invention provides the use of a sterile nutrient composition derived from the biomass of a culture of bacteria including methanotrophic bacteria, and optionally containing further nutrients, as a microorganism growth medium.

The bacterial culture used to produce the biomass is preferably at least 50%, more preferably at least 60%, especially at least 70%, in particular at least 75%, e.g. 75 to 95%, more particularly 75 to 80%, by weight methanotrophic bacteria (relative to the total bacterial weight).

Viewed from a further aspect the invention provides a method of culturing microorganisms which comprises bringing together a microorganism and a growth medium therefor, characterised in that said growth medium is or is prepared from a sterile nutrient composition derived from the biomass of a culture of bacteria including

methanotrophic bacteria, optionally with the addition of further nutrients.

Viewed from a yet further aspect the invention provides a microorganism growth substrate comprising a
5 sterile nutrient composition derived from the biomass of a culture of bacteria including methanotrophic bacteria, further containing at least one sterile nutrient, and optionally containing a diluent.

The biomass from which the growth medium or
10 substrate is prepared is preferably biomass generated from at least one species of methanotrophic bacteria and at least one species of heterotrophic bacteria, preferably grown in the same culture medium, e.g. using a loop reactor provided with methane, oxygen, ammonia,
15 and mineral feeds. Suitable combinations of bacteria for generating the biomass are described for example in WO 01/60974 the contents of which are incorporated by reference. One particularly suitable combination is *Methylococcus capsulatus* (Bath) (strain NCIMB 11132),
20 *Ralstonia* sp. DB3 (strain NCIMB 13287), *Aneurinibacillus* sp. DB4 (strain NCIMB 13288) and *Brevibacillus agri* DB5 (strain NCIMB 13289).

The biomass from the bacterial culture may be used directly (although generally after dewatering and
25 sterilization) or it may first be processed to break down the bacterial cells, e.g. by homogenization, hydrolysis or autolysis. Such treatments are described in WO01/60974 and British Patent Applications Nos. 0203306.6 and 0203307.4 filed 12 February 2002 which are
30 also incorporated herein by reference. (Copies of these two British Patent Applications are also filed herewith.) While homogenizate, hydrolysate and autolysate, especially dried homogenizate and more especially dried autolysate, of the bacterial biomass
35 are the preferred materials for the preparation of microorganism growth media according to the invention, precursor materials obtained by filtration (e.g. ultra

filtration) of the homogenized, autolysed or hydrolysed biomass, i.e. the liquid filtrate itself and the retentate, may also be used. Most preferred however is the dried autolysate.

5 The microorganism growth medium may be the bacterial biomass product itself or a composition containing the biomass product and further constituents, e.g. a liquid or non-liquid carrier or diluent (such as water, gel (e.g. agar gel), or a gellable liquid), and
10 materials such as minerals, carbon sources (such as saccharides (e.g. mono, di, oligo and polysaccharides, especially mono and disaccharides)), nitrogen sources (e.g. nitrates, proteins or protein fragments, ammonium compounds, oligopeptides, amino acids (especially
15 essential amino acids, e.g. tryptophan)), nucleic acids and nucleic acid fragments, lipids, etc. Particularly preferably the medium contains glucose and added nitrate and mineral salts (e.g. potassium, calcium, magnesium, sodium, molybdenum, iron, zinc, boron, cobalt, manganese
20 and nickel compounds), especially glucose. The composition as provided may be a sterile solid (e.g. particulate), a semi-solid or a liquid in ready to use or concentrate form. Especially preferably the composition as provided will be a sterile dry
25 particulate concentrate transformable into a growth medium by the addition of water or an aqueous gelling agent composition.

Wherein the composition contains added glucose, this is preferably in a dry mass basis weight ratio of
30 5:1 to 1:5 (especially 2:1 to 1:2) relative to the biomass deriving component. Where the composition contains added nitrate and mineral salts those preferably in a weight ratio of 0.01:1 to 0.2:1 (especially 0.05:1 to 0.1:1) relative to the biomass
35 deriving component. Where the composition as provided contains no added glucose and/or nitrate mineral salts, it is preferred that the preparation of the growth

medium involve addition of one or both such components in the weight ratios specified above.

5 The compositions of the invention are particularly suitable for use as growth substrates for heterotrophic microorganisms, especially heterotrophic algae, yeast or bacteria, in particular anaerobic bacteria such as lactobacilli, aerobic bacteria such as *E. coli*, and algae such as *Cryptocodinium cohnii*.

10 The invention will now be illustrated further with reference to the following non-limiting Examples.

EXAMPLE 1

Biomass Extracts

15 Methanotrophic and heterotrophic bacteria (*Methylococcus capsulatus* (Bath) (strain NCIMB 11132) *Ralstonia* sp. DB3 (strain NCIMB 13287), *Aneurinibacillus* sp. DB4 (strain NCIMB 13288) and *Brevibacillus agri* DB5 (strain NCIMB 13289)) were cultivated as described in WO01/60974 and
20 the resulting biomass harvested and treated as described in WO01/60974 to produce spray-dried homogenizate (hereinafter "BP Homogenizate"), as described in British Patent Application No. 0203306.6 to produce spray-dried hydrolysate (hereinafter "BP Hydrolysate"), and as
25 described in British Patent Application No. 0203307.4 to produce an autolysate (hereinafter "BP Autolysate"). Where the post-autolysis ultrafiltration and evaporation steps in the production of BP Autolysate are omitted, the product is referred to herein as "BP Crude
30 Autolysate". The product referred to as "BP Retentate" is an ultra-high-temperature treated biomass that was homogenized. The product referred to as "BP Permeate" corresponds essentially to the liquid by-product of the ultrafiltration step in the production of BP
35 Homogenizate. Microorganism growth media were produced by adding BP Homogenizate, BP Crude Autolysate, BP Autolysate, BP Retentate and BP Permeate to

demineralized water at a concentration of 1g/L. These media were then used either directly or with the addition of 0.1 g/L glucose and/or 32.4 mL/L Nitrate Mineral Salt medium (NMS).

5

NMS comprises:

1.0g KNO_3
0.2g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
10 1.0g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
0.1 mL trace element solution*
0.1 mL sodium molybdate solution (5g/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ in demineralized water)
0.1 mL EDTA solution (45g/L $\text{FeNaEDTA} \cdot 2\text{H}_2\text{O}$ in water)
15 water - to 1L
10 mL/L sterile phosphate buffer (35.6g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 26.0g KH_2PO_4 and water to 1L) was added.

*6.4g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
20 150 mg H_3BO_3
600 mg $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$
130 mg MnCl_2
100 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$
demineralized water - to 1L

25

All media were autoclaved before use.

An agar-based microorganism growth medium was also prepared containing:

30

32.2 g/L BP Autolysate
20.0 g/L glucose
34 mL/L NMS medium
14.0 g/L agar
35 demineralized water to 1L

This was autoclaved before use.

EXAMPLE 2

Microorganism Growth Tests

5 Aerobic and anaerobic, Gram positive and Gram negative
bacteria were grown in a shake flask using the liquid
growth media of Example 1 and, as controls, growth media
recommended for the bacterial strains. The optical
density of the cultures was monitored as an indicator of
10 the obtained bacterial growth (i.e. the "plateau" or
stationary phase with the highest number of cells). The
results are set out in Table 1 below.

Table 1

Bacterium	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Lactobacillus plantarum</i>	<i>Escherichia coli</i>
15 Characteristics	G(-), aerob	G(+), aerob	G(+), anaerob	G(-), aerob
Control Substrate	CASO	NB	MRS	TSB
BP Homogenisate	+	+++	+++	+
BP Autolysate	+++	+++	+++	+++
20 BP Crude autolysate	---	+++	+++	+++
BP Retentate	+	+++	+++	+++
BP Permeate	+	+	+++	---

25 --- : No combinations of the BP derivative were better
than control substrate.

+ : Some combinations of the BP derivative were as good
as the control substrate.

+++ : Some combinations of the BP derivative were
clearly better than control substrate.

30

For E. coli, BP Autolysate with added glucose clearly
provided the best growth. For L. plantarum, all
combinations of BP Autolysate clearly gave the best
growth. For P. aeruginosa, all combinations of BP
35 autolysate gave the best growth, in particular BP

Autolysate with added glucose. For *B. subtilis*, BP Autolysate with added glucose and both glucose and NMS added clearly gave the best growth.

5 EXAMPLE 3

Viability of Unknown Bacteria

Agar gels (PCA, MRS-agar, and BP Autolysate with agar(Example 1) pH 6.0 and 7.1) were spread with unknown
10 microorganisms taken from a sample of chopped meat. The cultures were incubated for 72 hours at 25°C and the total plate count was recorded. For BP Autolysate, log (CFU/g) was between 5 and about 6.6 while for MRS it was less than 1. For PCA log(CFU/g) was about 6.7, i.e.
15 barely higher.

EXAMPLE 4

Lactobacillus Viability

20 The lactobacillus strains *L. casei* ssp. *rhamnosus* (strain ATCC 7469), *L. delbruekii* ssp. *lactis* (strain ATCC 7830), *L. fermentum* (strain CCUG 30138), *L. gasseri* (strain ATCC 19992), and *L. plantarum* (strain ATCC 8014) were grown under aerobic conditions on MRS agar and BP
25 Autolysate agar (Example 1). In all cases the viability, measured as log(CFU/mL), was the same or greater for BP Autolysate. This was most pronounced for *L. delbruekii*. The same strains were also grown on these media under anaerobic conditions and again
30 viability was the same or better in all cases for BP Autolysate.

EXAMPLE 5

Production of Polyunsaturated Fatty Acids

35

Cryptocodinium cohnii (Seligo) Javornicky (strain ATCC 30772) was grown on a culture medium comprising 9g/L

- 5 glucose, 25g/L sea salt and 2g/L of either yeast extract (YE) or BP Autolysate in demineralized water. After two days of incubation, the cells were harvested and the total fatty acid, cell dry weight (CDW) and 22:6 (docosahexaenoic acid) contents were determined. The results are set out in Table 2 below.

Table 2

10

Culture Medium	CDW (g/L)	Lipid (%)	Lipid (g/L)	22:6 (%)	22:6 (g/L)
YE	3.2	12.0	0.39	36.1	0.139
BP Autolysate	4.2	7.9	0.33	40.9	0.135

- 15 Table 2 shows that the CDW and the percentage of the polyunsaturated fatty acid 22:6 was higher when BP Autolysate was used.

Claims:

1. The use of a sterile nutrient composition derived from the biomass of a culture of bacteria including methanotrophic bacteria, and optionally containing further nutrients, as a microorganism growth medium.
2. A method of culturing microorganisms which comprises bringing together a microorganism and a growth medium therefor, characterised in that said growth medium is or is prepared from a sterile nutrient composition derived from the biomass of a culture of bacteria including methanotrophic bacteria, optionally with the addition of further nutrients.
3. A microorganism growth substrate comprising a sterile nutrient composition derived from the biomass of a culture of bacteria including methanotrophic bacteria, further containing at least one sterile nutrient, and optionally containing a diluent.